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1. Walton et al., Journal of clinical endocrinology and metabolism. 2001 August. Vol. 86, No. 8, pp. 3675-85.
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Studies on the relative in-vitro biological potency of the naturally-occurring isoforms of intrapituitary follicle stimulating hormone

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In the present study, we analysed and compared the relative in-vitro biological activity of the various intrapituitary human follicle stimulating hormone (FSH) isoforms employing two different bioassay systems. FSH was fractionated by chromatofocusing (pH range 7.10 to <3.80) and the several isoforms isolated were quantified at multiple dose levels by three highly specific immunoassay systems: radioimmunoassay (RIA), enzyme-immunoassay (EIA) and immunoradiometric assay (IRMA), as well as by two in-vitro bioassays, one that measures the amount of oestrogen produced by rat granulosa cells in culture and the other that determines the amount of cAMP produced by a human fetal cell line (293) expressing the recombinant human FSH receptor. The relative in-vitro biological activity of each FSH isoform, expressed as the bioassay/immunoassay (B/I) activity ratio (B/RIA, B/EIA and B/IRMA ratios) varied with its elution pH value. Regardless of the immunoassay or bioassay method employed, less acidic FSH isoforms exhibited higher B/I ratios than their more acidic counterparts [B/RIA, B/EIA and B/IRMA ratios for isoforms with elution pH values >4.5 = 1.05 ± 0.13, 0.99 ± 0.10 and 1.15 ± 0.08 (rat oestrogen bioassay), and 2.75 ± 0.34, 2.20 ± 0.25 and 2.96 ± 0.35 (human cAMP production bioassay) respectively. Ratios for isoforms with pH values <4.5 = 0.71 ± 0.06, 0.47 ± 0.05 and 0.63 ± 0.06 (rat oestrogen assay), and 1.80 ± 0.26, 1.10 ± 0.09 and 1.44 ± 0.13 (cAMP assay) respectively (P <0.05 for isoforms with pH <4.5 compared with those isoforms with pH >4.5)]. Furthermore, statistically significant direct relationships between the B/RIA, B/EIA and B/IRMA ratios and the elution pH value of each isoform was identified by regression analysis [rat assay: r = 0.844, 0.800 and 0.780 (P <0.01); human assay: r = 0.730, 0.845 and 0.821 (P <0.01), for their corresponding B/RIA, B/EIA and B/IRMA ratios respectively]. The finding of significant differences in relative in-vitro biological potency among the various intrapituitary FSH isoforms strongly suggests that the shifts towards the production and secretion of more basic or acidic FSH molecules occurring in certain specific physiological conditions (e.g. puberty and menstrual cycle), may represent an important mechanism through which the anterior pituitary regulates gonadal function.

Key words: follicle stimulating hormone/FSH bioactivity/FSH glycoforms/granulosa cells/recombinant FSH receptor

Introduction

Follicle stimulating hormone (FSH) exists as a family of isohormones which have been identified within the anterior pituitary, serum and urine of several animal species, including man (Ulloa-Aguirre *et al.*, 1995a). The number and relative abundance of each FSH isoform will depend on the isolation technique employed for separation, the source of the sample and the physiological status of the donor at the time of tissue or sample collection (Ulloa-Aguirre *et al.*, 1988, 1995a; Dahl *et al.*, 1988; Wide, 1989). Since these isoforms have been separated principally on the basis of electrical charge, it seems that they differ from each other in their post-translationally determined carbohydrate composition, specifically their sialic acid content (Ulloa-Aguirre *et al.*, 1984; Blum *et al.*, 1985; Wide, 1989).

As a consequence of their structural differences, FSH isoforms differ in their ability to bind to target cell receptors, survive in the circulation and induce a biological response *in vivo* and *in vitro* (Chappel *et al.*, 1983; Blum and Gupta, 1985; Wide, 1986; Wide and Hobson, 1986; Ulloa-Aguirre *et al.*, 1992a). More acidic FSH isoforms (possessing a greater sialic acid content) from rodents and humans have considerably longer plasma half-lives but lower receptor-binding activity than their less acidic counterparts; on the contrary, less acidic variants disappear rapidly from the circulation and exhibit a three-fold higher receptor-binding/immunoactivity relationship than the more acidic forms (Chappel *et al.*, 1983; Blum and Gupta, 1985; Wide, 1986; Ulloa-Aguirre *et al.*, 1992a). However, the overall net in-vivo effect of the different FSH isoforms on biopotency, which depends on the interplay of several factors including receptor-binding affinity, signal transduction and circulating half-life (Bishop *et al.*, 1994, 1995; Chappel, 1995; Ulloa-Aguirre *et al.*, 1995a), is still unclear.

Several studies have attempted to analyse the in-vitro biological activity of human FSH isoforms with widely

differing results (Wide and Hobson, 1986; Ulloa-Aguirre *et al.*, 1992a; Burgon *et al.*, 1993; Simoni *et al.*, 1994). Some of these studies have clearly shown that the in-vitro biological activity/immunoactivity ratio (as an index of the relative in-vitro biopotency of individual isoforms) of FSH is lower for the more acidic and higher for the least acidic isoforms (Wide and Hobson, 1986; Ulloa-Aguirre *et al.*, 1992a). However, other studies have failed to demonstrate such a clear relationship between the apparent charge of the FSH molecule and its ability to bind its receptor and/or induce a biological response at the target cell level (Stanton *et al.*, 1992; Burgon *et al.*, 1993; Simoni *et al.*, 1994). Such discrepancies have been attributed to the use of receptor-binding assays, bioassays and immunoassays with different specificities, sensitivities, standard preparations of variable molecular composition and/or antibodies with different epitope specificity (Dahl and Stone, 1992; Burgon *et al.*, 1993; Simoni *et al.*, 1993,1994). Recent studies have unequivocally documented the occurrence of significant changes in FSH heterogeneity during certain physiological conditions including puberty and the menstrual cycle (Padmanabhan *et al.*, 1988; Wide, 1989; Wide and Bakos, 1993; Phillips and Wide, 1994; Zambrano *et al.*, 1995). Thus, clarification of isoform bioactivity is of critical importance to assign a functional significance to the existence of such a variety of isoforms for a single hormone. We therefore decided to re-examine in more detail the in-vitro biological activity of the various human FSH isoforms present in anterior pituitary extracts employing two different bioassays. FSH was fractionated by chromatofocusing and the capacity of the different isoforms to induce oestrogen synthesis by rat granulosa cells in culture as well as adenosine 3′,5′-monophosphate (cAMP) production by a human fetal kidney cell line expressing the recombinant human FSH receptor was studied.

Material and methods

Pituitary extracts

Adult human pituitaries, not selected by sex or age, were collected at autopsies performed after accidental deaths. The bodies were examined no later than 24 h post-mortem and were kept at 8°C within 3–4 h after death. The pituitary glands were stored frozen at –70°C until extracts were prepared. The anterior pituitary total glycoprotein extracts were obtained following the method of Jones *et al.* (1970). Extracts were kept at –70°C until the day of chromatofocusing. The study was approved by the human and animal research ethical committees of the Institute.

Chromatofocusing of pituitary glycoprotein extracts

Chromatofocusing of FSH present in pituitary glycoprotein extracts was performed according to the method described previously with some modifications (Ulloa-Aguirre *et al.*, 1990). To increase the resolution of the chromatofocusing separation, columns of polybuffer exchange resin (PBE-94; Pharmacia Fine Chemicals, Piscataway, NJ, USA) with larger dimensions (90×1.5 cm) than those previously used (Ulloa-Aguirre *et al.*, 1990, 1992a) were constructed and equilibrated with 15 bed volumes of starting buffer (0.025 M imidazole–HCl, pH 7.4). Subsequently, a highly concentrated pituitary glycoprotein extract (15–25 mg of immunoreactive FSH), which had previously been equilibrated with the eluent buffer [1:8 dilution of Polybuffer-74 (Pharmacia) in deionized water, pH 4.0] by chromatography on Sephadex G-25 (Pharmacia) was applied to the top of the column. Eluent buffer (5 ml) was run before sample application to avoid exposure of the sample proteins to pH extremes. Between 550 and 600 fractions (3 ml each) were collected at a flow rate of 1 ml/4 min at 4°C. The pH of each fraction was measured and when the pH of the column eluent stabilized at its lowest value, the eluent buffer was then replaced by a solution of 1 M NaCl to recover those proteins bound at the lower limiting pH (salt peak). Each fraction was divided into several aliquots which were stored frozen at –20°C until further analysis.

The amount of FSH was determined in 5–15 µl aliquots of each fraction by radioimmunoassay. Recoveries of FSH by this method were 79 ± 4% of the total amount applied to the column. After three chromatofocusing separations, fractions containing the greatest concentration of each immunoreactive FSH isoform (Figure 1) were separately pooled, transferred to dialysis membrane tubings (molecular weight cut-off 12 000; Spectrum Medical Industries, Los Angeles, CA, USA), dialysed at 4°C for 24 h against deionized water and thereafter against 0.01 M ammonium carbonate (pH 7.5) and freeze-dried. Each FSH isoform, or pool of neighbouring isoforms, was redissolved in phosphate (0.01 M)-buffered physiological (0.15 mol/l) saline (PBS; pH 7.4) and kept frozen at –70°C until measurement of FSH content.

Immunoassays of FSH

Radioimmunoassay

Purified human FSH [human FSH-I3; National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA] was iodinated by the lactoperoxidase-glucose oxidase method (Bex and Corbin, 1981). Following separation of protein-bound and free [^{125}I] by Sephadex G-100 column chromatography, [^{125}I]-labelled FSH was further purified by Concanavalin A chromatography (Pharmacia) as described by Dufau *et al.* (1972). The FSH radioimmunoassay was performed using reagents provided by the NIDDK; the reference preparation LER-907 (1 mg LER-907 = 53 IU 2nd International Reference Preparation) was employed to construct the standard curve. This standard exhibits a similar degree of charge heterogeneity to crude pituitary extracts as disclosed by chromatofocusing (Chappel *et al.*, 1986). The polyclonal antihuman FSH-6 at a final dilution of 1:250 000 was used as antiserum; this antiserum exhibits <0.1% cross-reactivity with highly purified human luteinizing hormone (LH) and undetectable reactivity with free α-subunit. To minimize the effects of interassay variations as well as to determine the degree of parallelism between the unknown samples and the FSH standard, all isoform concentrates prepared were analysed at multiple dose levels in the same assay run. Inter- and intra-assay coefficients of variation were <12% and <8% respectively, and the sensitivity was 0.24 mIU/tube.

Enzyme immunoassay (EIA) and immunoradiometric assay (IRMA)

The EIA of FSH was performed employing reagents provided by the World Health Organization (WHO) Collaborating Centre for Research and Reference Services in the Immunoassay of Hormones in Human Reproduction, London, UK. This assay employs two anti-FSH monoclonal antibodies directed against either the β- or the α-chain of the glycoprotein. The IRMA of FSH was performed using a commercial [^{125}I]-IRMA kit (Immunochem™; ICN Biomedicals, Inc, Costa Mesa CA, USA); this immunometric assay uses a monoclonal antibody directed against the α-subunit and a polyclonal/monoclonal antibody mix directed against the β-subunit. In both immunoassays, the standard curve was constructed using LER-907 as standard. Each sample was

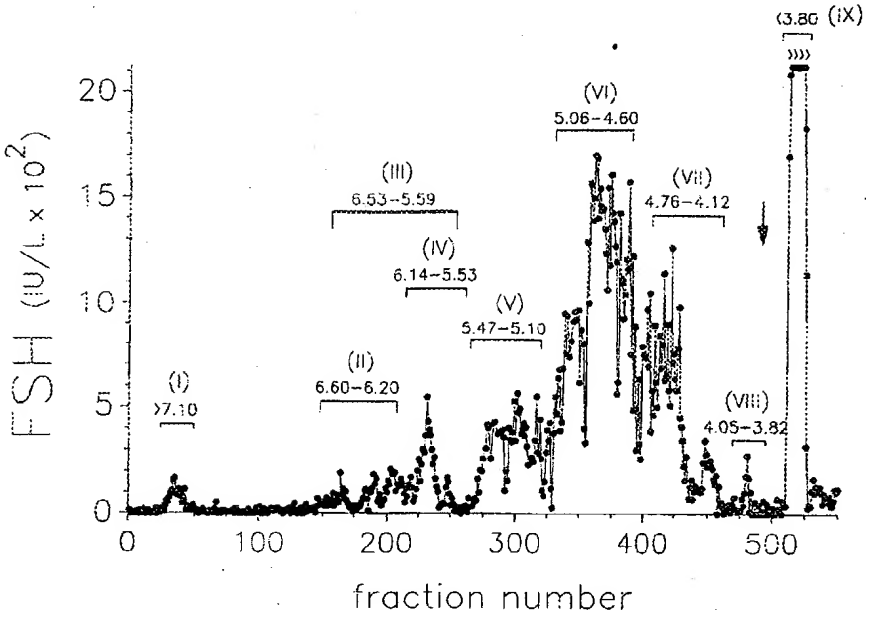

Figure 1. Representative profile of pH distribution of immunoreactive follicle stimulating hormone (FSH) after chromatofocusing of an anterior pituitary glycoprotein extract. The elution pH values of the various FSH isoforms (I to IX) are indicated. Isoform concentrate III comprised both concentrate II and isoform concentrate IV. The arrow marks the application of 1 M NaCl to the chromatofocusing column.

assayed at 6–8 different dilutions to detremine the degree of parallelism with the LER-907 standard. Cross reactivity with free α-subunit, LH, thyroid-stimulating hormone (TSH) and human chorionic gonadotrophin (HCG) in these systems was <1.0%. To minimize the effects of interassay variations, all isoform concentrates were analysed in the same batch. Inter and intra-assay coefficients of variation for both assay systems were <8 and <5% respectively.

In-vitro bioassays of FSH

Oestrogen production by rat granulosa cells: aromatization bioassay (GAB)

The capacity of each FSH isoform concentrate to induce aromatization of androgen *in vitro* was assessed following the method described by Jia and Hsueh (1985). Inter- and intra-assay coefficients of variation were <15 and <8% respectively. The amount of oestrogen produced *in vitro* was determined by radioimmunoassay using an antiserum against oestrone (Ulloa-Aguirre *et al.*, 1992a). Because of the significant cross-reactivity of this antiserum with 17-β oestradiol (~22%), results are expressed as total oestrogens produced by the granulosa cells in culture. All samples from a single bioassay were analysed for oestradiol content in the same batch; the mean inter- and intra-assay coefficients of variation were <11 and <6% respectively.

cAMP production by human fetal cells expressing the FSH receptor

The human embryonic kidney-derived 293 cell line transfected with the human FSH receptor cDNA was a generous gift of Dr. Aaron J.W. Hsueh, Stanford University, CA, USA. The origin, handling, ligand specificity and biochemical properties of the recombinant human FSH receptor expressed by this cell line have been described elsewhere (Tilly *et al.*, 1992). Transfected 293 cells (2×10^5 cells/culture dish) were exposed to increasing doses of each isoform concentrate in the presence of 0.125 mM 3-isobutyl-*1*-methylxanthine (Sigma Chemical Co, St. Louis, MO, USA) for 24 h at 37°C. After incubation, total (intra- and extracellular) cAMP concentrations were determined by radioimmunoassay after acetylation of the samples and cAMP standards. The assay employed 2-*O*-monosuccinyl cAMP tyrosylmethyl ester (Sigma) iodinated by the chloramine-T method as the labelling ligand and the CV-27 cAMP antiserum (NIDDK) at a final dilution of 1:150 000. After incubation at 4°C for 24 h, antibody-bound and free cAMP were separated by ethanolic precipitation followed by centrifugation at 1200 *g* at 4°C. The sensitivity of the assay was 4 fmol/tube and the inter- and intra-assay coefficients of variation were <12 and <6% respectively.

In both assays, the standard curve was constructed using LER-907 as the FSH standard. Each sample was tested for in-vitro bioactivity at 8–10 different doses ranging from 0.5–65 mIU/culture dish, in triplicate incubations. The relative in-vitro biological activity of FSH was calculated considering each dose analysed in four separate assays and it is expressed as the bioassay/immunoassay (B/I) activity ratio, the ratio of activity in the in-vitro bioassay relative to the activity in the radioimmunoassay (B/RIA), EIA (B/EIA) and IRMA (B/IRMA).

Statistical analysis

Tests for parallelism among the slopes generated by the different FSH preparations (LER-907 and isoform concentrates) in the immunoassays and bioassays of FSH were performed following the method of DeLean *et al.* (1978). One-way analysis of variance (ANOVA) was used to determine differences between the apparent in-vitro biological activity of the FSH isoforms. When differences existed *t*-tests were used to determine their statistical significance. Linear regression analysis was performed to determine the degree and significance of the association between the elution pH value of the isoforms and their corresponding in-vitro B/I ratios. $P<0.05$ was considered to be statistically significant.

Results

Separation of pituitary glycoprotein extracts on high-resolution chromatofocusing columns revealed the presence of multiple distinct peaks of FSH immunoactivity with pH values ranging from >7.10 to <3.80. Two peaks of FSH immunoactivity were identified at each end of the pH window; the first appeared in fractions containing those proteins that passed

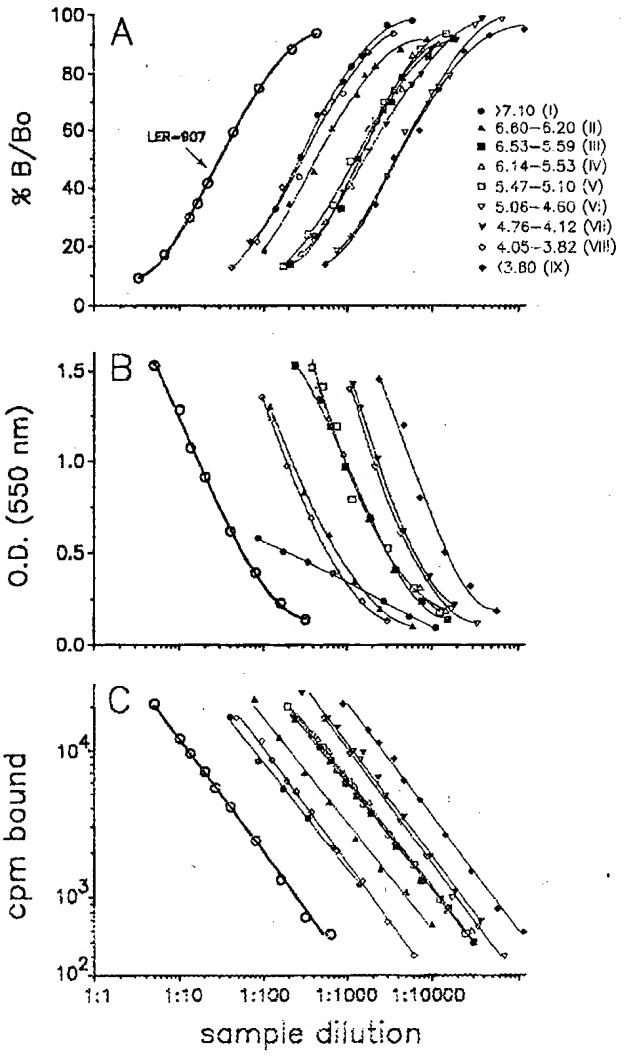

Figure 2. Representative dose-response curves of the different follicle stimulating hormone (FSH) isoform concentrates (I to IX) in the radioimmunoassay (**A**), enzyme immunoassay (**B**) and immunoradiometric assay (**C**) systems. Similar results were obtained from three separate assays.

through the column unrestricted (pH >7.10), whereas the second, with an elution pH value of <3.8–3.0, was identified at the end of chromatofocusing after the application of 1 M NaCl (Figure 1). Fractions containing the highest concentrations of immunoactive FSH within a single peak or closely neighbouring peaks were pooled in nine groups as shown in Figure 1, concentrated as described previously and then utilized in the immuno- and bioassay studies.

All the pools displaced [^{125}I]-labelled FSH from the antibody in a parallel fashion when analysed by radioimmunoassay (Figure 2A); simultaneous curve fitting of the dose-response curves revealed no significant differences among the slopes generated by the FSH standard (LER-907) and the several pools of isoforms fractionated by chromatofocusing. Similar results were obtained when the amount of immunoreactive FSH present in each concentrate was determined by EIA (Figure 2B) and IRMA (Figure 2C), with the exception of the least acidic isoform (present in pool I) which was not accurately quantified in the EIA system due to significant non-concordance between this preparation and the corresponding standard curve. When compared with the radioimmunoassay, EIA slightly overestimated the amount of FSH present in concentrates III, V, and VII to IX, whereas IRMA underestimated the amount of FSH present in pools I, IV, VI and VIII but overestimated those contained in concentrates VII and IX. EIA underestimated the amount of FSH present in isoform concentrates III to IX when compared with IRMA. These differences in antibody recognition led to slight variations in the calculated B/I ratio for the same isoform, reaching in some cases statistical significance (Table I).

In-vitro bioactivity of the FSH isoforms

All FSH concentrates were able to induce significant dose-dependent oestrogen production when analysed by the rat granulosa cell aromatization bioassay (Figure 3); their corresponding dose-response curves were parallel regardless of the method (radioimmunoassay, EIA or IRMA) employed to determine the FSH doses to be challenged [mean ± SD of the slope factors: LER-907, 3.10±0.20; isoform concentrates I to IX, 2.71±0.22 (range 2.43 ± 0.30 to 3.01 ± 0.28; not significant)]. The results obtained with GAB showed that FSH isoforms in pools II to VI (elution pH value 6.60 to 4.60) exhibited higher in-vitro B/I ratios than the more acidic FSH isoforms (VII to IX; Table I and Figure 4). In fact, if those isoforms with elution pH values >7.10 (I) and <3.80 (IX) were excluded, since their exact pH could not be determined by the chromatofocusing method employed, there was a statistically significant direct relationship between the B/I ratios of the isoforms and their corresponding mean elution pH value [r = 0.844, 0.800 and 0.780 for the B/RIA, B/EIA and B/IRMA ratios respectively (P <0.01)]. The least acidic FSH isoforms (concentrated in pool I) exhibited a B/RIA ratio similar to that of isoforms with elution pH values ≤5.06 and a B/IRMA ratio similar to that of isoforms with pH values 5.47–4.60. Likewise, depending on the immunoassay method employed to quantitate the amount of the most acidic isoform (contained in pool IX), the B/I ratios of this variant were intermediate between those of isoforms VI and VII (elution pH value 5.06 to 4.12) or isoforms VII and VIII (pH 4.76 to 3.82) (Table I).

Human fetal 293 cells exposed to each isoform concentrate produced cAMP in an FSH dose-dependent manner (Figure 5). The slope values of the dose-response curves yielded by this assay were not significantly different [mean slope value for LER-907 = 1.46 ± 0.12, isoforms I to IX, 1.30 ± 0.10 (range 1.02 ± 0.17 to 1.65 ± 0.10); not significant]. The relative in-vitro bioactivity of the less acidic FSH isoforms was significantly higher than that exhibited by the more acidic forms (r = 0.730, 0.845 and 0.821 for the B/RIA, B/EIA and B/IRMA ratio of isoforms II to VIII and their corresponding mean elution pH value respectively; P <0.01); all B/I ratios were higher in isoforms with a pH value >4.5 (Figure 4). In this assay, however, the least acidic FSH isoform consistently exhibited the highest in-vitro bioactivity (Table I). Other discrepancies between the two bioassay systems were also evident for isoform concentrates V to VII (Table I). In general,

Table I. Bioactivity/immunoactivity (B/I) ratio of the different isoforms of follicle stimulating hormone (FSH) isolated from anterior pituitary glycoprotein extracts. Values are means ± SEM derived from four independent bioassays

Isoform (pH)	FSH B/I ratio					
	Oestrogen production (rat assay)			cAMP production (human assay)		
	B/RIA	B/EIA	B/IRMA	B/RIA	B/EIA	B/IRMA
I (>7.10)	0.86 ± 0.02^{ac}	—	$1.03 \pm 0.05^{ac\P}$	3.9 ± 0.1^{a}	—	$4.7 \pm 0.2^{a\P}$
II (6.60–6.20)	1.32 ± 0.1^{b}	1.31 ± 0.1^{a}	1.31 ± 0.1^{b}	3.5 ± 0.2^{b}	3.4 ± 0.2^{a}	3.2 ± 0.2^{b}
III (6.53–5.59)	$1.19 \pm 0.05^{bc\dagger}$	$0.90 \pm 0.04^{b\S}$	1.18 ± 0.05^{ab}	$2.7 \pm 0.2^{c\dagger}$	$2.1 \pm 0.1^{b\S}$	2.7 ± 0.2^{c}
IV (6.14–5.53)	1.14 ± 0.1^{cd}	$1.03 \pm 0.05^{b\S}$	1.24 ± 0.08^{b}	2.2 ± 0.1^{de}	$2.0 \pm 0.1^{b\S}$	$2.6 \pm 0.1^{c\P}$
V (5.47–5.10)	$1.01 \pm 0.02^{ad\dagger}$	$0.67 \pm 0.02^{d\S}$	$0.92 \pm 0.03^{c\P}$	$3.2 \pm 0.2^{b\dagger}$	$2.1 \pm 0.1^{b\S}$	2.9 ± 0.2^{cb}
VI (5.06–4.60)	$0.87 \pm 0.02^{ae\dagger}$	$0.97 \pm 0.02^{b\S}$	$1.14 \pm 0.01^{ab\P}$	1.4 ± 0.1^{f}	$1.5 \pm 0.1^{c\S}$	$1.8 \pm 0.1^{d\P}$
VII (4.76–4.12)	$0.72 \pm 0.03^{ef\dagger}$	$0.35 \pm 0.01^{e\S}$	$0.48 \pm 0.02^{d\P}$	$2.5 \pm 0.05^{ce\dagger}$	$1.2 \pm 0.03^{c\S}$	$1.7 \pm 0.04^{d\P}$
VIII (4.05–3.82)	$0.64 \pm 0.01^{f\dagger}$	$0.56 \pm 0.01^{df\S}$	$0.72 \pm 0.02^{e\P}$	$0.89 \pm 0.02^{g\dagger}$	$0.77 \pm 0.02^{d\S}$	$1.0 \pm 0.02^{e\P}$
IX (<3.80)	0.77 ± 0.1^{ef}	0.48 ± 0.05^{ef}	0.65 ± 0.1^{de}	$2.1 \pm 0.02^{d\dagger}$	$1.3 \pm 0.04^{c\S}$	$1.8 \pm 0.1^{d\P}$

RIA = radioimmunoassay; EIA = enzyme immunoassay; IRMA = immunoradiometric assay.
Means identified by different superscript letters in the same column are significantly different ($P < 0.05$).
$^{\dagger}P < 0.05$ B/RIA versus B/EIA; $^{\S}P < 0.05$ B/EIA versus B/IRMA; $^{\P}P < 0.05$ B/IRMA versus B/RIA.

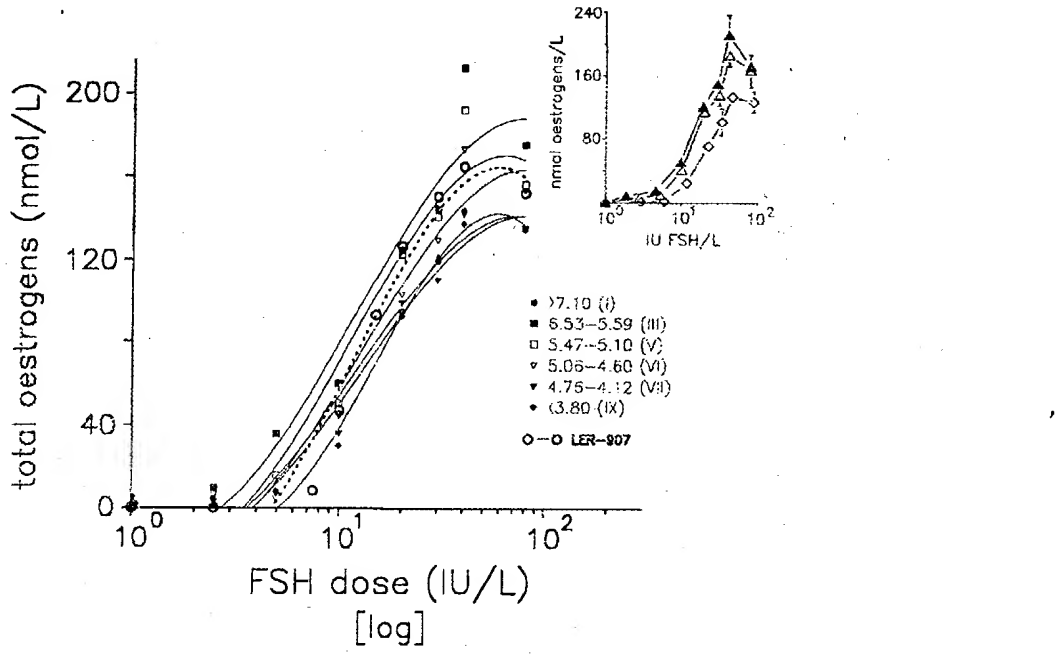

Figure 3. The ability of increasing doses of follicle stimulating hormone (FSH) isoform concentrates I, III, V, VI, VII and IX to induce oestrogen production by rat granulosa cells in culture. The dose is expressed in terms of the LER-907 standard (NIDDK) as measured by radioimmunoassay. The dispersion around each mean is omitted for clarity. *Inset:* The dose response curves of FSH isoform concentrates II (▲), IV (△) and VIII (◇).

the behaviour of the most acidic component in the human assay was similar to that observed in the GAB.

Discussion

In the present study, we have examined the capacity of intrapituitary FSH isoforms to induce a quantitatively measurable response at the post-receptor level. The results showed that all isoforms, after separation by a charge-based procedure, were able to induce significant dose-dependent responses in each of two separate bioassay systems. The first was a primary cell assay that assesses the ability of FSH to induce aromatization of androgen by cultured rat granulosa cells, and the second used a cell line transfected with the human FSH receptor which had been optimized to produce cAMP as an end point. A B/I ratio was established as an index of the relative in-vitro biological activity of each FSH isoform. To properly interpret the results, the same pituitary standard, possessing a charge heterogeneity similar to that of crude pituitary extracts (Chappel *et al.*, 1986), was used to construct the standard curve in all assay systems employed. Likewise, to overcome some of the potential problems related to differences in antibody recognition or epitope specificity, the amount of isoform in each in-vitro bioassay was defined using three different immunoassay systems which used either polyclonal or monoclonal antibodies. Although all isoforms exhibited parallel dose-dependent curves in the radioimmunoassay, IRMA and EIA systems (with the exception of isoform I in

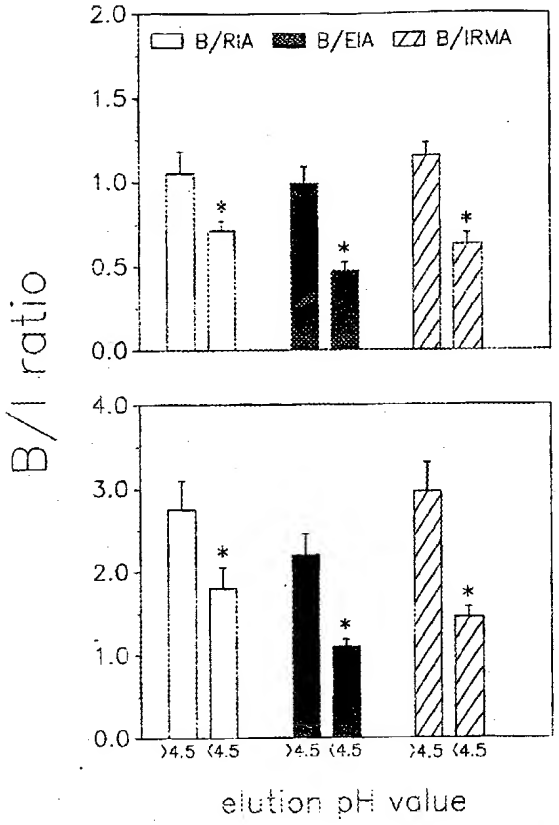

Figure 4. The ratio of biological to immunological (B/I) activity of follicle stimulating hormone (FSH) exhibited by isoforms with elution pH values >4.5 and <4.5 in the rat granulosa cell aromatization bioassay (top) and the cAMP production human assay (bottom). *Pairs in which there are statistically significant differences ($P < 0.05$). Data derived from four independent bioassays (mean ± SEM).

the EIA), significant variations in B/I ratios within the same isoform pool were detected, implying that some of the immunoassay systems recognized certain isoforms more or less accurately than the others. However, in spite of these differences in antibody recognition towards certain FSH isoforms, a trend was apparent in which the less acidic isoforms exhibited higher B/I ratios than the more acidic forms. Whether this close relationship between FSH isoform charge and B/I ratio was the result of a general inability of each of the immunoassay systems employed to accurately identify the less acidic/sialylated isoforms or whether these isoforms are genuinely more bioactive cannot be determined by the present study. It is currently difficult to draw definitive conclusions concerning the intrinsic in-vitro biological activity of the human FSH isoforms. However, differences in oligosaccharide structures (which are essential for receptor binding and signal transduction) are the distinguishing characteristic of most gonadotrophin isoforms (Ulloa-Aguirre *et al.*, 1988, 1995a). A series of observations has shown that rodent, monkey, and human FSH as well as human choriogonadotrophin and rat and human luteinizing hormone share the same relationship between charge and the in-vitro biopotency of their corresponding isoforms (Miller *et al.*, 1983; Khan *et al.*, 1984; Hattori *et al.*, 1985; Wide and Hobson, 1986; Ulloa-Aguirre *et al.*, 1988, 1990, 1992a; present study). This suggests that increased bioactivity may be the cause of the greater B/I ratios exhibited by the less acidic intrapituitary FSH isoforms.

The least acidic FSH isoform, although reliably quantified by radioimmunoassay and IRMA, was barely detected by the EIA. It has been previously shown that these particular FSH molecules are poorly sialylated (Ulloa-Aguirre *et al.*, 1984), exhibit more basic pH values (Dahl *et al.*, 1988) and bear oligosaccharide structures markedly different to the more acidic isoforms (see below). Whether these variations in immunoactivity are dependent on the specific carbohydrate composition of these basic molecules or on the presence of a heterogeneous peptide component (Ulloa-Aguirre *et al.*, 1995a) remains to be clarified.

In both the human and the rat bioassay systems, and regardless of the immunoassay method employed to quantify the isoforms, the highest in-vitro bioactivity was exhibited by the less acidic isoforms and the lowest by their more acidic/sialylated couterparts. However, some differences between the results generated by the two bioassays were apparent. In the human assay, the B/I ratios obtained for each isoform were higher (2 to 3 times) than those yielded by the rat assay, a finding which may be explained by the greater homology between the structurally heterogeneous human FSH ligands tested and their corresponding receptor in the 293 cell system (Mulder *et al.*, 1994). On the other hand, the least acidic FSH molecules (present in pool 1) consistently exhibited the highest B/I ratios in the human assay whereas in the GAB they showed a lower activity than that observed for isoforms with pIs 6.60 to 5.53. This, and other similar discrepancies between the apparent *in vitro* bioactivites shown by the same isoforms in the two assays, may be secondary to the different abilities of the individual bioassay systems to identify subtle aspects of the molecular structure of FSH, mainly those related to the carbohydrate chains attached to the protein core. Recent lectin-binding studies have indicated that, in addition to their lower sialic acid content, less acidic FSH isoforms bear more high mannose- and hybrid-type oligosaccharides, whereas the more acidic variants contain greater amounts of more complex carbohydrate residues (Ulloa-Aguirre *et al.*, 1992b; Creus *et al.*, 1996). These variations in oligosaccharide composition may account for differences in the affinity of the isoforms for the human and the rat FSH receptor and/or their capability to activate it and establish signal transduction (Sairam and Bhargavi, 1985; Bishop *et al.*, 1994; Davis *et al.*, 1995). Alternatively, these apparent discrepancies may relate to the particular biological features of the rat granulosa cell and the receptor-transfected 293 cell systems employed in the present study. The human assay has been maximized for cAMP production and thus neither steps post-cAMP nor involvement of other second messenger systems which may eventually lead to agonistic or antagonistic outcomes are considered (Dahl *et al.*, 1988; Flores *et al.*, 1990; Padmanabhan *et al.*, 1991; Minegishi *et al.*, 1995). In fact, dissociations between cAMP and end product relationships have been recently demonstrated in primary cell cultures utilizing deglycosylated FSH analogues (Padmanabhan *et al.*, 1991; Ulloa-Aguirre *et al.*, 1995a).

These results are in accordance with other recent findings

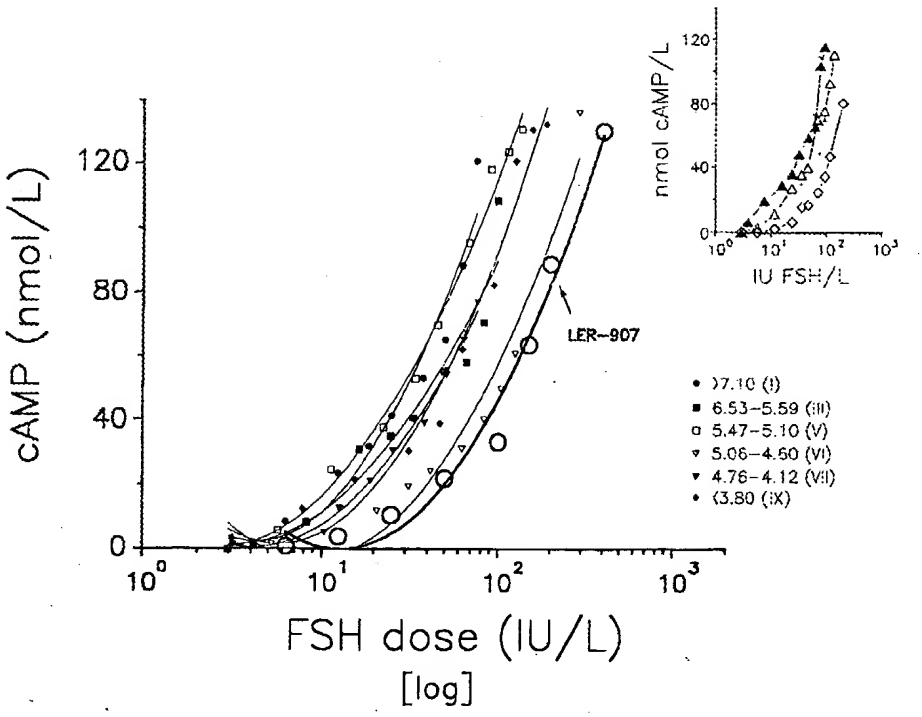

Figure 5. The ability of increasing doses of follicle stimulating hormone (FSH) isoform concentrates I, III, V, VI, VII and IX to induce cAMP production by 293 cells transfected with the recombinant human FSH receptor. The dose is expressed in terms of the LER-907 standard (NIDDK) as measured by radioimmunoassay. The dispersion around each mean is omitted for clarity. *Inset:* The dose response curves of FSH isoform concentrates II (▲), IV (△) and VIII (◇).

showing a clear association between the relative in-vitro B/I ratios and the charge borne by various recombinant human FSH preparations, urinary FSH compounds and their corresponding isoforms (Cerpa-Poljak *et al.*, 1993; Flack *et al.*, 1994, Lambert *et al.*, 1995; Ulloa-Aguirre *et al.*, 1995b, 1996). Interestingly, in one of these studies (Flack *et al.*, 1994) the least acidic recombinant variant also showed a significantly decreased biological activity as disclosed by GAB. The overall findings concurrently suggest that assays employing final products (e.g. oestrogen) as end points to assess the bioactivity of either natural or recombinant FSH isoforms, may be more suitable than the cAMP production-based human assays in establishing the clinical significance of changes in the relative distribution of secreted isoforms during specific physiological conditions (Padmanabhan *et al.*, 1988; Wide and Bakos, 1993; Phillips and Wide, 1994; Zambrano *et al.*, 1995) and in determining the potential effects of differing molecular structures in recombinant FSH on the various biological functions of the gonad.

The present findings are in contrast with those from other investigators who were unable to detect a clear relationship between the receptor-binding potency and/or *in vitro* bioactivity and the charge of the pituitary FSH isoforms (Stanton *et al.*, 1992; Burgon *et al.*, 1993; Simoni *et al.*, 1994). However, in one of those studies (Simoni *et al.*, 1994), chromatofocusing columns with much lower resolving capacity were employed to fractionate the various FSH isoforms, whereas in the study of Burgon *et al.*, (1993), only those isoforms recovered within a relatively narrow isoelectric point (pI) range (3.5–5.29) were considered. In addition, neither of these two studies accurately established parallelism between the dose-response curves generated by the various isoforms and the particular standard preparations used in the bio- and immunoassays. Finally, Stanton *et al.*, (1992), whose study was also restricted to isoforms with pI values 3.5–5.29, observed that the dose-response curves of FSH isoforms with pI values 4.15–5.29 were not parallel to those of the more acidic isoforms or the FSH standard used in the heterologous assay employed to calculate the radioreceptor specific activity of the isoforms. It is well known that the establishment of parallelism between the dose-response curves of standards and unknowns is critical for a reliable estimation of relative potencies or B/I ratios (DeLean *et al.*, 1978; Chappel, 1995; Ulloa-Aguirre *et al.*, 1995a). In fact, Blum *et al.* (1985) observed that the dose-response relationships of the more acidic pituitary rat FSH isoforms differed considerably from both the less acidic forms and the reference preparation used to construct the standard curve; the less acidic isoforms exhibited B/I ratios equal to 1.0 over the entire concentration range analysed, whereas the more acidic variants showed a progressive increase in this ratio with values <1.0 and >3.0 at low and high concentrations respectively. Since in the foregoing studies the methods for determining the potencies or B/I ratios of FSH isoforms were not clearly validated, it is very difficult to draw definitive conclusions concerning the significance of their findings.

We and others have previously shown that almost all stored FSH isoforms may be released from the pituitary gland with few or no modifications in their number and pH values and that the charge distribution of the circulating isoforms changes according to the phase of the menstrual cycle (Padmanabhan *et al.*, 1988; Wide and Bakos, 1993; Zambrano *et al.*, 1995).

Specifically, the secretion of isoforms with pH values >4.5 is significantly increased at times of both high oestradiol output and enhanced pituitary sensitivity to GnRH (*e.g.* during the periovulatory phase) (Zambrano *et al.*, 1995). The finding that their intrapituitary counterparts display an increased relative *in vitro* biopotency (Ulloa-Aguirre *et al.*, 1992a; present study) strongly suggests that the shift towards the production and secretion of less acidic/sialylated FSH molecules during this cycle phase may be an important mechanism to regulate the intensity of the FSH stimulus during the final steps of follicular maturation. The fact that the less acidic isoforms disappear rapidly from the circulation (Wide, 1986; Ulloa-Aguirre *et al.*, 1992a,b) does not necessarily mean that under physiological conditions their presence may be biologically irrelevant. Less acidic isoforms possess *per se* considerable bioactivity and even if small quantitites reach the target cell they still might be able to induce a significant biological response. Evidence for this possibility has been provided for desialylated human choriogonadotrophin, which has been shown to be a full agonist *in vivo* in the monkey, capable of stimulating a full testicular response over 6 h despite being cleared from the circulation within 15 min (Liu *et al.*, 1989). Therefore, the signal transduction system at the receptor level may be capable of achieving full biological effect despite low receptor occupancy or dramatically shortened exposure to the stimulus. Additional studies on the in-vivo bioactivity of circulating FSH isoforms are still required to further support this hypothesis.

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